

An overview of the sexual dimorphism in *Echiniscus* (Heterotardigrada, Echiniscoidea), with the description of *Echiniscus masculinus* sp. nov. (the *virginicus* complex) from Borneo

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Abstract

Members of the genus *Echiniscus* C.A.S. Schultze, 1840 are mostly unisexual, with thelytokously reproducing females. Therefore, every newly described dioecious species in the genus is particularly interesting. Here, we describe *Echiniscus masculinus* sp. nov. from Gunung Kinabalu, the highest peak of Borneo and the entire Southeast Asia. The new species belongs in the predominantly parthenogenetic *E. virginicus* complex, and its females are confusingly similar to females of the pantropical *E. lineatus* Pilato et al., 2008, another member of this group. However, genetic evidence and noticeable sexual dimorphism clearly delineate the new species. Males of *E. masculinus* sp. nov. are unlike females in the body proportions, cuticular sculpturing, and appendage configuration. The new discoveries provide a justification to review the current knowledge about evolution and forms of sexual dimorphism within *Echiniscus*.

Key Words

bisexual, clavae, dioecious, Echiniscidae, endemic, Gunung Kinabalu, limno-terrestrial life cycle, tropics

Introduction

A swiftly increasing number of tardigrade species is currently at circa 1300 species (Guidetti and Bertolani 2005; Degma and Guidetti 2007; Degma et al. 2009–2019), which have already approached the conservative estimate of Bartels et al. (2016). This number has also exceeded the mean estimate of circa 1150 (upper 95% CI >2100) limno-terrestrial tardigrade species based on a protocol by Mora et al. (2011). Recent works have included DNA barcoding in modern tardigrade taxonomy, disclosing numerous species complexes in various phylogenetic lineages of the phylum (e.g. Stec et al. 2018; Guidetti et al. 2019; Cesari et al. 2020). On the other hand, many tardigrade groups contain a significant number of dubious or synonymic taxa (e.g. Gąsiorek et al. 2019b). Within the class Heterotardigrada, the greatest progress in solving taxonomic and phylogenetic problems has been made regarding a fascinating group of armoured limno-terrestrial

tardigrades, the family Echiniscidae (Kristensen 1987; Jørgensen et al. 2011, 2018; Vicente et al. 2013; Vecchi et al. 2016; Gąsiorek et al. 2018a, 2018b, 2019b; Cesari et al. 2020). Recently, Gąsiorek et al. (2019a) demonstrated synonymy within the *Echiniscus virginicus* complex, reducing the number of valid species from five to four, and for the first time presenting an integrative evidence for a pantropical tardigrade species. Currently, only one member of this group, *E. clevelandi* (Beasley 1999), is dioecious.

Gunung Kinabalu, together with the Crocker Range located farther south, constitute the highest prominence in the northern part of Borneo. Due to the remarkable geological and climatic conditions, an altitudinal zonation of flora is present on Gunung Kinabalu (Kitayama 1992), which is a characteristic of many high mountain peaks in the Indomalayan region (van Steenis 1984; Ohsawa et al. 1985). Consequently, these mountains harbour unparalleled animal diversity associated with rich plant vegeta-

tion, even for the extraordinarily speciose faunae of the Malay Archipelago (Lohman et al. 2011; de Bruyn et al. 2014). On the other hand, as in many tropical areas, some animal groups remain barely known. This is the case with tardigrades, the subject of only two Bornean papers (Pilato et al. 2004; Gąsiorek 2018). Given the recent explosion of hidden species diversity in several tardigrade genera, it is more than likely that Bornean rainforests hold numerous undescribed tardigrade species.

In this contribution, by using morphological and phylogenetic methods, we describe *Echiniscus masculinus* sp. nov. from a high elevation in Gunung Kinabalu. The new species sheds light on the evolution of the *E. virginicus* complex and raises questions about the prevalent type of speciation (sympatric vs allopatric) in this group. Finally, the sexual dimorphism within *Echiniscus* is compared to that of other echiniscids, and the apparent morphological stasis in females of the *E. virginicus* complex is discussed.

Methods

Sample collection and specimen preparation

A total of 52 animals representing the new species was extracted from a moss sample collected in Northern Borneo by Maciej Barczyk on 29 June 2016 (sample code MY.026). The air-dried sample, stored in a paper envelope, was rehydrated in water for several hours, and the obtained sediment was poured into Petri dishes to search for microfauna under a stereomicroscope with dark field illumination. Individuals isolated from the sample were used for two types of analysis: imaging in light microscopy (morphology and morphometry; 44 specimens) and DNA sequencing + phylogenetics (eight specimens).

Imaging, morphometrics, and terminology

Individuals for light microscopy and morphometry were first air-dried on microscope slides, and then mounted in a small drop of Hoyer's medium and examined under a Nikon Eclipse 50i phase contrast microscope (PCM) associated with a Nikon Digital Sight DS-L2 digital camera. All figures were assembled in Corel Photo-Paint X6, ver. 16.4.1.1281. For deep structures that could not be fully focused in a single light microscope photograph, a series of 2–12 images was taken every circa 0.1 μm and then assembled into a single deep-focus image. All measurements are given in micrometres (μm) and were performed under PCM. Structures were measured only if they were not damaged and if their orientations were suitable. Body length was measured from the anterior to the posterior end of the body, excluding the hind legs. The *sp* ratio is the ratio of the length of a given structure to the length of the scapular plate (Dastyh 1999). Morphometric data were handled using the Echiniscoidea ver. 1.3

template available from the Tardigrada Register, <http://www.tardigrada.net/register> (Michalczyk and Kaczmarek 2013). The terminology follows Kristensen (1987) and subsequent changes proposed in Gąsiorek et al. (2019b). For qualitative differential diagnoses, species descriptions and amendments of the four taxa constituting the *Echiniscus virginicus* group were studied (Riggin 1962; Moon and Kim 1990; Beasley 1999; Abe et al. 2000; Pilato et al. 2008; Kaczmarek and Michalczyk 2010; Gąsiorek et al. 2019a).

Genotyping and phylogenetics

The DNA was extracted from eight individual animals following a Chelex 100 resin (Bio-Rad) extraction method by Casquet et al. (2012) with modifications described in detail in Stec et al. (2015). All specimens were mounted in water on temporary slides and examined under PCM before DNA extraction to ensure correct taxonomic identifications. One hologenophore cuticle (Pleijel et al. 2008) was retrieved from an Eppendorf tube, mounted on a permanent slide, and deposited in the Institute of Zoology and Biomedical Research in Kraków. We sequenced four nuclear and one mitochondrial DNA fragments: the small and the large ribosome subunit 18S rRNA and 28S rRNA (918 bp and 728 bp, respectively), the internal transcribed spacers ITS-1 and ITS-2 (642 and 484 bp, respectively), and the cytochrome oxidase subunit I COI (632 bp). All fragments were amplified and sequenced according to the protocols described in Stec et al. (2015); primers and original references for specific PCR programmes are listed in Table 1. Sequences were aligned using default settings of MAFFT7 (Katoh et al. 2002; Katoh and Toh 2008) under G-INS-i strategy. Uncorrected pairwise distances were calculated using MEGA7 (Kumar et al. 2016) and are included as the Suppl. material 2.

To ensure that the topologies of the trees reconstructed on the basis of genetic markers were identical, we calculated Bayesian inference (BI) marginal posterior probabilities using MrBayes ver. 3.2 (Ronquist and Huelsenbeck 2003) for each of the three markers (COI, ITS-1, and ITS-2) separately. Random starting trees were used, and the analysis was run for ten million generations, sampling the Markov chain every 1000 generations. An average standard deviation of split frequencies of <0.01 was used as a guide to ensure that the two independent analyses had converged. The program Tracer ver. 1.3 (Rambaut et al. 2014) was then used to ensure that Markov chains had reached stationarity and to determine the correct 'burn-in' for the analysis, which was the first 10% of generations. The ESS values were >200, and a consensus tree was obtained after summarizing the resulting topologies and discarding the 'burn-in'. Trees were rooted on *Echiniscus succineus*. Clades recovered with a posterior probability (PP) between 0.95 and 1.00 were considered well supported, those with a PP between 0.90 and 0.94 were considered moderately supported, and those with a low-

Table 1. Primers and references for specific protocols for amplification of the five DNA fragments sequenced in the study.

DNA fragment	Primer name	Primer direction	Primer sequence (5'–3')	Primer source	PCR programme*
18S rRNA	18S_Tar_Ff1	forward	AGGCGAAACCGCGAATGGCTC	Stec et al. (2018)	Zeller (2010)
	18S_Tar_Rr2	reverse	CTGATCGCCTTCGAACCTCTAACTTTCG	Gąsiorek et al. (2017)	
28S rRNA	28S_Eutar_F	forward	ACCCGCTGAACTTAAGCATAT	Gąsiorek et al. (2018a)	Mironov et al. (2012)
	28SR0990	reverse	CCTTGGTCCGTGTTTCAAGAC	Mironov et al. (2012)	
ITS-1	ITS1_Echi_F	forward	CCGTCGCTACTACCGATTGG	Gąsiorek et al. (2019a)	Welnicz et al. (2011)
	ITS1_Echi_R	reverse	GTTTCAGAAAACCTGCAATTACAG		
ITS-2	ITS3	forward	GCATCGATGAAGAACGCAGC	White et al. (1990)	
	ITS4	reverse	TCCTCCGCTTATTGATATGC		
COI	bcdF01	forward	CATTTTCHACTAAYCATAARGATATTGG	Dabert et al. (2008)	
	bcdR04	reverse	TATAAACYTCDGGATGNCCAAAAA		

* All PCR programmes are also provided in Stec et al. (2015).

er PP were considered unsupported. All final consensus trees were viewed and visualized using FigTree ver. 1.4.3 (available at: <https://tree.bio.ed.ac.uk/software/figtree>).

Data deposition

Raw morphometric data are placed as the Suppl. material 1 and in the Tardigrada Register under <http://www.tardigrada.net/register/0062.htm>. Type DNA sequences are deposited in GenBank.

Results

Taxonomic account

Phylum Tardigrada Doyère, 1840
 Class Heterotardigrada Marcus, 1927
 Order Echiniscoidea Richters, 1926
 Family Echiniscidae Thulin, 1928
 Genus *Echiniscus* C.A.S. Schultze, 1840

Echiniscus masculinus sp. nov.

<http://zoobank.org/99CA96E7-D111-4E07-A0A2-4133F54755C9>

Figures 1–3, Tables 2–5

Description. Mature females (i.e. from the third instar onwards; measurements and statistics in Table 2). Body cylindrical, orange with minute red eyes present in live specimens; colours disappearing soon after mounting in Hoyer's medium. *Echiniscus*-type cephalic papillae (secondary clavae) and (primary) clavae; cirri growing out from bulbous cirrophores (Figure 1A). The body appendage configuration is *A-C-D-D^d-E*, with all trunk appendages formed as spines or spicules. All usual trunk appendages always symmetrical and smooth. Spine *C^d* rudimentarily developed in two females (one with an asymmetrical spicule [2 µm], the other normally formed [8 µm]).

Dorsal plates with the mixed type of sculpturing, with an evident layer of endocuticular pillars visible as black dots under PCM, and an upper layer of greyish epicuticular matrix forming the ornamented pattern together with pseudopores, enhanced as dark belts on the anterior por-



Figure 1. Morphology of *Echiniscus masculinus* sp. nov. (PCM). **A.** Adult female (holotype, dorsolateral view); **B.** Juvenile (paratype, dorsolateral view); **C.** Subcephalic plates; **D.** Genital plates enclosing male gonopore; **E.** First leg pair with claws and spine I. All scale bars in µm.

tions of the paired segmental plates (Fig. 1A). Generally, the epicuticular sculpture is poorly developed and gives way to large pillars, especially on the cephalic and scapular plates, and also on the central portion of the median plate I and centroposterior portions of segmental plates. The cephalic plate is relatively large whereas the cervical (neck) plate is barely demarcated from the scapular plate,

Table 2. Measurements [in μm] of selected morphological structures of the adult females of *Echiniscus masculinus* sp. nov. mounted in Hoyer's medium. N – number of specimens/structures measured, RANGE refers to the smallest and the largest structure among all measured specimens; SD – standard deviation; *sp* – the proportion between the length of a given structure and the length of the scapular plate.

Character	N	Range		Mean		SD		Holotype	
		μm	<i>sp</i>	μm	<i>sp</i>	μm	<i>sp</i>	μm	<i>sp</i>
Body length	10	159–192	432–492	175	453	11	21	178	444
Scapular plate length	10	32.6–43.7	–	38.8	–	3.2	–	40.1	–
Head appendages lengths									
Cirrus <i>internus</i>	9	9.7–15.5	25.1–38.8	12.6	32.4	2.3	4.9	14.6	36.4
Cephalic papilla	10	5.9–7.8	15.1–19.2	6.7	17.3	0.5	1.3	6.4	16.0
Cirrus <i>externus</i>	8	12.3–18.8	37.7–47.0	16.6	42.7	1.9	3.7	18.8	46.9
Clava	10	4.7–6.4	11.4–17.1	5.5	14.2	0.6	1.6	5.6	14.0
Cirrus A	10	23.3–42.3	69.6–105.5	32.8	84.7	4.7	10.7	33.1	82.5
Cirrus A/Body length ratio	10	15%–24%	–	19%	–	3%	–	19%	–
Body appendages lengths									
Spine C	10	10.9–21.6	33.4–56.3	16.6	43.0	3.1	8.3	15.1	37.7
Spine D	10	11.2–21.6	29.5–57.9	16.0	41.3	3.1	8.3	13.8	34.4
Spine D ^d	10	2.9–16.8	8.9–45.0	11.7	30.1	4.1	10.9	9.7	24.2
Spine E	10	13.6–23.3	33.9–60.7	18.6	48.3	2.5	8.2	13.6	33.9
Spine on leg I length	10	3.0–3.9	8.0–11.0	3.4	8.8	0.3	0.9	3.3	8.2
Papilla on leg IV length	10	3.6–5.3	9.9–12.9	4.4	11.3	0.6	1.1	4.6	11.5
Number of teeth on the collar	9	8–12	–	10.1	–	1.3	–	9	–
Claw I heights									
Branch	8	8.8–10.7	23.5–27.6	9.7	25.3	0.6	1.7	9.7	24.2
Spur	8	2.2–3.2	6.7–8.5	2.8	7.3	0.3	0.6	2.7	6.7
Spur/branch height ratio	8	24%–33%	–	29%	–	2%	–	28%	–
Claw II heights									
Branch	9	8.4–10.4	21.5–25.9	9.4	24.4	0.6	1.4	10.0	24.9
Spur	9	2.1–3.1	6.4–8.2	2.8	7.1	0.3	0.5	2.6	6.5
Spur/branch height ratio	9	25%–33%	–	29%	–	3%	–	26%	–
Claw III heights									
Branch	10	8.4–10.2	22.7–26.2	9.5	24.5	0.6	1.2	9.9	24.7
Spur	10	2.0–3.1	6.1–7.2	2.6	6.6	0.3	0.4	2.9	7.2
Spur/branch height ratio	10	24%–31%	–	27%	–	2%	–	29%	–
Claw IV heights									
Branch	7	9.4–12.1	24.9–30.3	10.9	27.4	0.9	2.4	?	?
Spur	7	2.3–3.2	6.1–8.6	3.0	7.4	0.3	0.9	?	?
Spur/branch height ratio	7	24%–29%	–	27%	–	1%	–	?	–

formed only as thin grey belt without pillars. The scapular plate large, with additional lateral sutures separating narrow rectangular lateral portions with poorly developed pillars. Paired segmental plates divided into a smaller, much narrower anterior and a dominant posterior part by a smooth, wide transverse stripe (Fig. 1A). The caudal (terminal) plate with short incisions and fully developed epicuticular layer. Median plate I unipartite, whereas median plate II divided into weakly defined parts, with a wide rhomboidal smooth space between them (Fig. 1A). Median plate III small but with a well-developed epicuticular layer. Ventral cuticle with minute endocuticular pillars distributed throughout the whole venter, and a pair of oval subcephalic (Fig. 1C) and trapezoid genital plates. Sexpartite gonopore placed between genital plates, and a trilobed anus between legs IV.

Pedal plates I–III absent, pedal plate IV developed as a dark matrix without pillars, bearing a typical dentate collar (Figure 1A). Distinct pulvini on all legs (Fig. 1A). A small spine on leg I (Fig. 1E) and a papilla on leg IV present. Claws IV slightly higher than claws I–III (Table 2). External claws on all legs smooth (Figure 1E). Internal claws with large spurs positioned at circa 1/3 of the claw height and bent downwards.

Buccal apparatus short, with a rigid, stout tube and a spherical pharynx. Stylet supports absent.

Mature males and sexually dimorphic traits (i.e. from the third instar onwards; measurements and statistics in Tables 3, 4). Generally resembling females, but a closer observation reveals two qualitative differences (body appendage configuration and dorsal plate sculpturing) and numerous morphometric dissimilarities between males and females (all summarised in Table 4). Densely punctuated areas in the central leg portions present (Fig. 2A). Male genital plates are always clearly visible (of identical shape as female plates), and dark densely arranged pillars are present in the entire genital zone, extending between the plates (Fig. 1D).

Juveniles (i.e. the second instar, measurements and statistics in Table 5). Clearly smaller than adult females and males, with the body appendage configuration A-C-D-D^d-E. Endocuticular pillars well developed in all plates, the largest pillars present in the posterior portion of the scapular plate and in the central part of the caudal (terminal) plate. Epicuticular ornamented pattern absent, although lighter and darker parts of the scapular plate can be distinguished under PCM (Fig. 1B), constituting presumably the developing epicuticular layer.

Larvae. Unknown.

Table 3. Measurements [in μm] of selected morphological structures of the adult males of *Echiniscus masculinus* sp. nov. mounted in Hoyer's medium. N – number of specimens/structures measured, RANGE refers to the smallest and the largest structure among all measured specimens; SD – standard deviation; *sp* – the proportion between the length of a given structure and the length of the scapular plate.

Character	N	Range		Mean		SD		Allotype	
		μm	<i>sp</i>	μm	<i>sp</i>	μm	<i>sp</i>	μm	<i>sp</i>
Body length	10	142–170	464–527	161	493	9	23	167	527
Scapular plate length	10	30.3–35.7	–	32.6	–	1.5	–	31.7	–
Head appendages lengths									
Cirrus <i>internus</i>	10	10.2–19.2	31.0–58.9	15.3	47.2	2.3	7.7	15.0	47.3
Cephalic papilla	10	7.7–9.3	23.4–30.0	8.6	26.6	0.6	2.2	8.6	27.1
Cirrus <i>externus</i>	10	16.0–21.0	47.3–67.3	18.8	57.8	1.6	6.0	17.5	55.2
Clava	10	6.1–7.5	19.2–22.8	6.8	20.8	0.4	1.1	6.1	19.2
Cirrus A	8	28.4–36.2	84.6–111.0	31.9	98.0	2.8	9.1	30.0	94.6
Cirrus A/Body length ratio	8	18%–24%	–	20%	–	2%	–	18%	–
Body appendages lengths									
Spine C	10	19.9–26.9	63.7–77.9	23.1	70.9	2.3	5.5	24.7	77.9
Spine D	10	17.6–29.7	54.0–83.2	23.0	70.4	3.4	8.5	25.0	78.9
Spine E	10	19.4–30.5	59.1–92.7	24.5	75.1	4.1	12.0	27.7	87.4
Spine on leg I length	10	2.0–3.7	6.5–11.3	3.1	9.6	0.5	1.4	2.8	8.8
Papilla on leg IV length	10	3.8–5.3	12.4–16.2	4.6	14.2	0.5	1.1	4.1	12.9
Number of teeth on the collar	9	7–12	–	9.4	–	1.7	–	12	–
Claw I heights									
Branch	10	8.4–10.7	26.5–33.0	9.4	28.9	0.7	2.1	8.4	26.5
Spur	10	2.2–3.1	6.9–9.9	2.7	8.3	0.3	0.8	2.2	6.9
Spur/branch height ratio	10	23%–32%	–	29%	–	3%	–	26%	–
Claw II heights									
Branch	9	8.4–10.4	24.9–32.1	9.2	28.4	0.6	2.5	8.6	27.1
Spur	9	1.9–2.7	5.8–8.9	2.4	7.5	0.3	1.0	2.6	8.2
Spur/branch height ratio	9	20%–31%	–	26%	–	3%	–	30%	–
Claw III heights									
Branch	8	8.5–10.1	25.8–31.4	9.2	28.3	0.6	2.0	8.7	27.4
Spur	8	2.3–2.8	7.0–8.5	2.5	7.7	0.2	0.5	2.3	7.3
Spur/branch height ratio	8	24%–30%	–	27%	–	2%	–	26%	–
Claw IV heights									
Branch	4	9.5–10.4	28.1–34.0	10.1	31.1	0.4	3.2	?	?
Spur	4	2.7–3.1	8.3–9.2	2.9	8.8	0.2	0.4	?	?
Spur/branch height ratio	4	26%–30%	–	28%	–	2%	–	?	–

Table 4. Sexual dimorphism in qualitative and quantitative traits in *Echiniscus masculinus* sp. nov., with results of one-tailed Student's *t*-tests in case of overlapping ranges in morphometric traits (all statistically significant at the α -level of $p_{\text{BH}} < 0.05$ adjusted with the Benjamini-Hochberg correction).

Qualitative traits	♀♀	♂♂	Remarks
Body appendage configuration	A-C-D-D ^d -E	A-C-D-E	a single male had an asymmetrically developed spine D ^d [8 μm long]
Cuticular sculpturing	epicuticular ornamentation poor	epicuticular ornamentation pronounced	compare Figures 1, 2A, B and 3A, but see also Figures 2C and 3B for an atypically poor sculpturing in a male
Quantitative traits	♀♀: $\bar{x} \pm \text{SD}$, N = 10	♂♂: $\bar{x} \pm \text{SD}$, N = 10	t, p
Body proportions: <i>bs</i> ratio	0.54–0.57 (= body larger and plump)	0.48–0.49 (= body smaller and slender)	non-overlapping ranges; see also Fig. 2
Body length	175 \pm 11	161 \pm 9	$t_{18} = 3.27$; $p = 0.002$
Scapular plate length	38.8 \pm 3.2	32.6 \pm 1.5	$t_{18} = 5.51$; $p < 0.001$
Head appendages lengths			
Cephalic papilla	17.3 \pm 1.3	26.6 \pm 2.2	$t_{18} = 11.47$; $p < 0.001$
Clava	14.2 \pm 1.6	20.8 \pm 1.1	$t_{18} = 10.44$; $p < 0.001$
Body appendage lengths			
Spine C	43.0 \pm 8.3	70.9 \pm 5.5	$t_{18} = 8.90$; $p < 0.001$
Spine D	41.3 \pm 8.3	70.4 \pm 8.5	$t_{18} = 7.75$; $p < 0.001$
Spine E	48.3 \pm 8.2	75.1 \pm 12.0	$t_{18} = 5.79$; $p < 0.001$
Claw branch heights			
Claw I	25.3 \pm 1.7	28.9 \pm 2.1	$t_{16} = 3.99$; $p < 0.001$
Claw II	24.4 \pm 1.4	28.4 \pm 2.5	$t_{16} = 4.36$; $p < 0.001$
Claw III	24.5 \pm 1.2	28.3 \pm 2.0	$t_{16} = 5.01$; $p < 0.001$

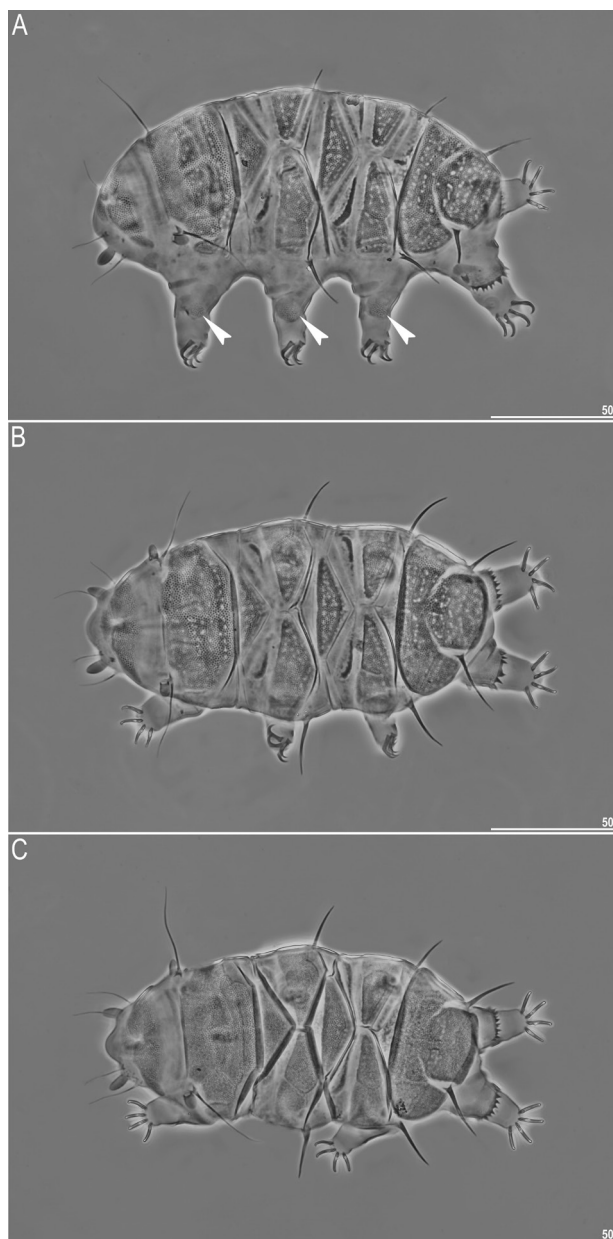


Figure 2. Morphology of males of *E. masculinus* sp. nov. (PCM). **A.** allotype (dorsolateral view, arrowheads indicate areas with densely packed pillars in legs); **B.** paratype with fully developed sculpturing (dorsal view); **C.** paratype with poorly developed epicuticular layer of sculpturing (dorsal view). See Table 4 for the phenotypic comparison between females and males. All scale bars in µm.

Eggs. Up to two round, yellow eggs per exuvia were found.

Genetic markers and phylogenetic position. The 18S rRNA, 28S rRNA and ITS-2 were characterised by single haplotypes (GenBank accession numbers: MT106621, MT106620, MT106622, respectively), but three haplotypes were detected in the case of ITS-1 (MT106623–5), and five in COI (MT106223–7). All three DNA-based phylogenetic reconstructions revealed *E. masculinus* sp. nov. as the sister species to the clade *E. lineatus* + *E. vir-*

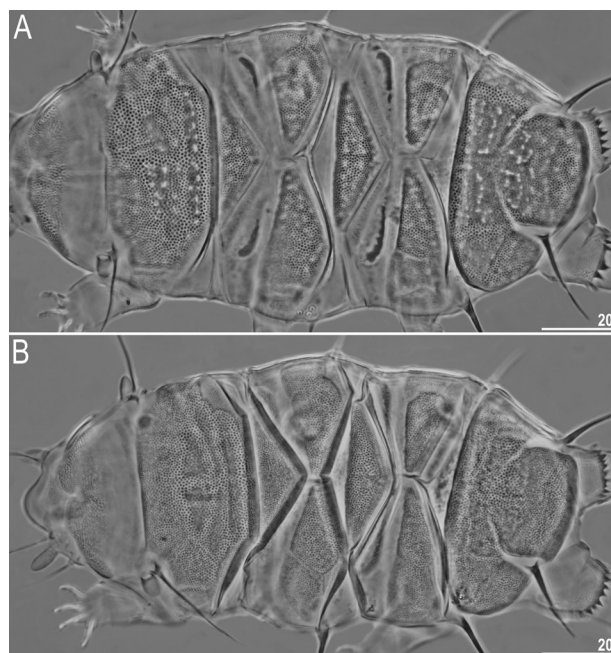


Figure 3. Close-up on the details of sculpturing of *E. masculinus* sp. nov. (PCM). **A.** evident epicuticular layer, endocuticular pillars of various sizes; **B.** remnants of epicuticular layer on the scapular and caudal (terminal) plates, endocuticular pillars densely packed and of equal, minute size. All scale bars in µm.

ginicus with a maximum support (Fig. 4). The divergence between the new species and the other two congeners was notably larger in COI compared to the ITS markers (compare Fig. 4A and 4B, C). The differences are congruent with the *p*-distances (see SM.2).

Type material. Holotype (mature female, slide MY.026.05), allotype (mature male, slide MY.026.07) and 42 paratypes on slides MY.026.01–09. Moreover, one voucher specimen (hologenophore) mounted on the slide MY.026.14. In total: 21 females, 14 males, and nine juveniles. Slides MY.026.01–07 are deposited in the Institute of Zoology and Biomedical Research, Jagiellonian University, Poland; slide MY.026.08 (4♀♀, 3♂♂, one juvenile) is deposited in the Natural History Museum of Denmark, University of Copenhagen, Denmark; slide MY.026.09 (4♀♀, 2♂♂, 2 juveniles) is deposited in the Catania University, Sicily, Italy. Found together with a new species of *Echiniscus* and a new species of *Pseudechiniscus* (descriptions in preparation).

Type locality. Ca 6°05'N, 116°32'E, ca 3500 m a.s.l.: Malaysia, Borneo, Sabah, Gunung Kinabalu; subalpine vegetation zone with single *Leptospermum* and *Rhododendron ericoides* bushes, moss on a stunted tree trunk.

Etymology. From Latin *masculinus* = male (an adjective in the nominative singular). The name underlines the presence of males in the new species, in contrast to closely related parthenogenetic *E. lineatus* and *E. virginicus*.

Table 5. Measurements [in μm] of selected morphological structures of the juveniles of *Echiniscus masculinus* sp. nov. mounted in Hoyer's medium. N – number of specimens/structures measured, RANGE refers to the smallest and the largest structure among all measured specimens; SD – standard deviation; *sp* – the proportion between the length of a given structure and the length of the scapular plate.

Character	N	Range		Mean		SD	
		μm	<i>sp</i>	μm	<i>sp</i>	μm	<i>sp</i>
Body length	5	115–148	431–477	129	454	13	18
Scapular plate length	5	26.0–34.4	–	28.4	–	3.6	–
Head appendages lengths							
Cirrus <i>internus</i>	5	7.4–12.3	27.1–35.8	8.8	30.7	2.0	3.5
Cephalic papilla	5	3.8–6.4	13.1–19.6	4.9	17.3	1.0	2.7
Cirrus <i>externus</i>	4	8.8–14.2	33.3–41.3	10.8	38.0	2.3	3.4
Clava	5	3.7–5.4	13.7–16.7	4.3	15.2	0.7	1.2
Cirrus A	5	19.5–28.6	74.1–83.5	22.8	80.1	3.5	3.7
Cirrus A/Body length ratio	5	16%–19%	–	18%	–	1%	–
Body appendages lengths							
Spine C	5	8.1–20.3	30.8–59.0	12.9	44.6	4.6	10.7
Spine D	5	7.4–17.5	28.5–50.9	11.6	39.9	4.1	9.3
Spine D ^d	5	7.1–16.1	27.0–46.8	10.4	35.9	3.4	7.3
Spine E	5	10.8–18.0	40.2–52.3	12.7	44.3	3.0	4.9
Spine on leg I length	4	1.9–2.7	7.2–9.1	2.2	7.9	0.4	0.9
Papilla on leg IV length	5	3.2–3.8	10.8–14.4	3.4	12.2	0.3	1.5
Number of teeth on the collar	5	7–8	–	7.6	–	0.5	–
Claw I heights							
Branch	5	6.3–9.3	24.0–27.0	7.3	25.6	1.2	1.2
Spur	5	1.5–2.7	5.2–8.1	1.9	6.8	0.5	1.2
Spur/branch height ratio	5	21%–31%	–	27%	–	4%	–
Claw II heights							
Branch	4	6.1–6.8	23.2–25.0	6.5	24.0	0.3	0.9
Spur	4	1.4–1.9	5.4–7.2	1.7	6.2	0.2	0.8
Spur/branch height ratio	4	22%–31%	–	26%	–	4%	–
Claw III heights							
Branch	4	6.3–8.9	23.0–25.9	7.1	24.3	1.2	1.2
Spur	4	1.7–2.5	5.8–7.3	2.0	6.7	0.4	0.6
Spur/branch height ratio	4	25%–29%	–	28%	–	1%	–
Claw IV heights							
Branch	4	6.7–9.1	25.4–27.7	7.6	26.3	1.0	1.0
Spur	4	1.8–2.8	6.8–8.5	2.2	7.7	0.4	0.8
Spur/branch height ratio	4	25%–33%	–	29%	–	4%	–

Differential diagnosis. There are four known members of the *E. virginicus* complex: *E. clevelandi* Beasley, 1999, *E. hoonsooi* Moon & Kim, 1990, *E. lineatus* Pilato et al., 2008, and *E. virginicus* Riggan, 1962 (Gašiorek et al. 2019a). *Echiniscus masculinus* sp. nov. can be differentiated from (body appendage configuration given collectively for both sexes):

1. *E. clevelandi*, recorded from China, the only other dioecious representative of this group, by the body appendage configuration (*A-C-D-(D^d)-E* in *E. masculinus* sp. nov. vs *A-B-C-C^d-D-D^d-E* in *E. clevelandi*) and dorsal sculpturing (faint and poorly visible epicuticular layer with pseudopores in *E. masculinus* sp. nov. vs well-developed epicuticular layer with bright and large pores in *E. clevelandi*; see Pilato et al. 2008).
2. *E. hoonsooi*, recorded from Korea, by the body appendage configuration (*A-C-D-(D^d)-E* in *E. masculinus* sp. nov. vs *A-(C)-(D)-E* in *E. hoonsooi*), homomorphic spurs on all legs (heteromorphic spurs I–III and IV in *E. hoonsooi*; see Abe et al. 2000), and by the presence of males.
3. *E. lineatus*, distributed widely in the tropical and subtropical zone, by the body appendage configuration

(*A-C-D-(D^d)-E* in *E. masculinus* sp. nov. vs *A-(B)-C-C^d-D-D^d-E* in *E. lineatus*), and by the presence of males.

4. *E. virginicus*, native to the eastern Nearctic realm, by the body appendage configuration (*A-C-D-(D^d)-E* in *E. masculinus* sp. nov. vs *A-(B)-C-C^d-D-D^d-E* in *E. virginicus*), dorsal plate sculpturing (pseudopores in *E. masculinus* sp. nov. vs pores in *E. virginicus*), and by the presence of males.

Discussion

The *Echiniscus virginicus* complex contains species with well-defined geographical ranges: *E. lineatus* is pantropical, *E. clevelandi* and *E. hoonsooi* are known from Far East Asia, and *E. virginicus* has been recorded only from the Nearctic (Gašiorek et al. 2019a). Phylogenetic analyses inferred the new species as sister to the clade *E. lineatus* + *E. virginicus*, with the latter two more closely related to each other than to *E. masculinus* sp. nov. (Fig. 4). This is surprising for two reasons: the same place of origin of *E. masculinus* sp. nov. and *E. lineatus*, the tropics, as both occur only there, and the morphological similarity of these two species, since they both have pseudopores.

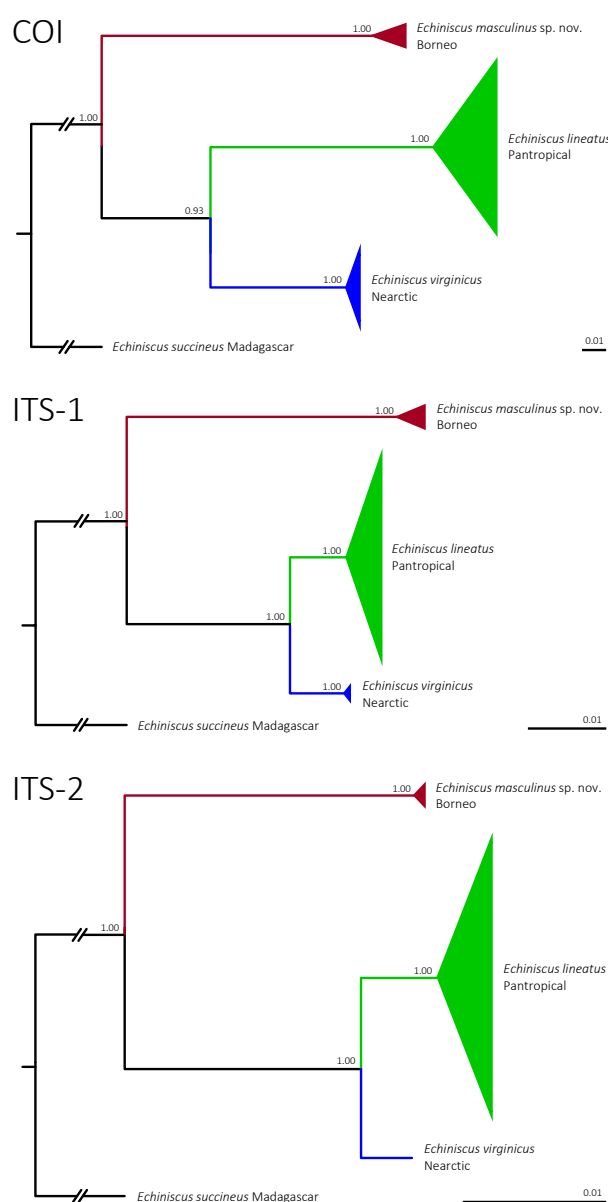


Figure 4. Bayesian phylogenetic trees showing the relationships between members of the *E. virginicus* complex; *E. succineus* was used as an outgroup, and branches within species-specific clades were collapsed. Bayesian posterior probability values are given above tree branches. Phylogenetic analyses were run on the subsequent DNA markers to assure that the tree topology was congruent: COI, ITS-1, and ITS-2.

As it is generally assumed that dioecy is ancestral, and parthenogenetic thelytoky is an advanced character within Echiniscidae (e.g. Kristensen 1987), the presence of males within populations of *E. masculinus* sp. nov. is probably a retained plesiomorphy of the entire complex. Given that the new species is described from a very peculiar habitat, namely a prominent mountain peak with high levels of endemism characterising many groups of animals (Merckx et al. 2015), the isolated locality suggests a contracted, relictual geographic range of *E. masculinus* sp. nov. and its potentially restricted area of occurrence (only Gunung Kinabalu or maybe also other high mountains of Borneo).

In contrast to arthrotardigrades, usually ancestrally dioecious (Fontoura et al. 2017), echiniscoidean taxa are more diversified in terms of reproductive modes and many groups embrace both parthenogenetic and dioecious species. Echiniscoididae and Oreellidae are bisexual (Kristensen and Hallas 1980; Dastych et al. 1998; Møbjerg et al. 2016), but sexual dimorphism is not well-marked in either of the two. The first observations on sexual dimorphism within Echiniscidae were documented by Dastych (1987) and Kristensen (1987). At present, males have been reported for 14 echiniscid genera: *Antechiniscus* (Claxton 2001), *Barbaria* (Miller et al. 1999; Michalczyk and Kaczmarek 2007), *Bryodelphax* (Gąsiorek and Degma 2018), *Claxtonia* (Kaczmarek and Michalczyk 2002; Mitchell and Romano 2007), *Cornechiniscus* (Dastych 1979), *Diploechiniscus* (Vicente et al. 2013), *Hypechiniscus* (Kristensen 1987), *Mopsechiniscus* (Dastych 2001), *Novechiniscus* (Rebecchi et al. 2008), *Proechiniscus* (Kristensen 1987), *Pseudechiniscus* (Cesari et al. 2020), *Stellariscus* (Gąsiorek et al. 2018b), *Testechiniscus* (Gąsiorek et al. 2018a), and *Echiniscus*. Sexual dimorphism can be obvious, as in *Mopsechiniscus*, or restricted to different gonopore shapes (e.g. in *Cornechiniscus*). Until now, males have been reliably discovered only in 11 *Echiniscus* spp. (Degma et al. 2009–2019): *E. clevelandi* (the *virginicus* complex), *E. curiosus* Claxton, 1996 and *E. merokensis* Richters, 1904 (the *merokensis* complex), *E. duboisi* Richters, 1902 and *E. siticulosus* Gąsiorek & Michalczyk, 2020 (the *spinulosus* complex), *E. ehrenbergi* Dastych & Kristensen, 1995 and *E. rodnae* Claxton, 1996 (the *testudo* complex), *E. jamesi* Claxton, 1996 (the *granulatus* complex), *E. lentiferus* Claxton & Dastych, 2017 (the *quadrispinosus* complex), *E. marleyi* Li, 2007 (the *blumi*–*canadensis* complex), *E. nepalensis* Dastych, 1975 (the *lapponicus* complex). The differences between the sexes are often minor (Dastych 1975; Dastych and Kristensen 1995; Miller et al. 1999), but some authors emphasised notable disparities in morphometric traits (Beasley 1999; Claxton 1996; Claxton and Dastych 2017; Gąsiorek and Michalczyk 2020). These encompass mainly differences in body proportions, and dimensions of claws, cephalic and trunk appendages (Claxton 1996; Gąsiorek and Michalczyk 2020). The sex ratio varies greatly even between populations of a single species (Miller et al. 1999), indicating that there may be seasonal variations in the presence of males within *Echiniscus* populations, as was observed for other micrometazoans (Gilbert and Williamson 1983).

Originally, the “Gondwanan” hypothesis was postulated to explain the distribution of dioecious *Echiniscus* spp. (Miller et al. 1999). In fact, except for the cosmopolitan *E. merokensis* and East Palaearctic *E. marleyi*, other dioecious *Echiniscus* spp. inhabit exclusively post-Gondwanan lands. Additionally, males are generally absent or present in almost negligible proportions in European and Central Asian populations of *Echiniscus* (Jørgensen et al. 2007; Guil and Giribet 2009). The evolutionary causes of this phenomenon are, however, still unknown.

The sexual dimorphism of *E. masculinus* sp. nov., evidenced in both quantitative and qualitative traits (Table 4) is interesting in the context of usually poorly marked sexual differences in dioecious *Echiniscus* spp., and the fact that females of *E. lineatus*, *E. virginicus*, and *E. masculinus* sp. nov. are confusingly similar to each other. In fact, females are a good example of profound evolutionary stasis in morphology, which led, for example, to a description of a synonymous species in the complex (*E. dariae* synonymised with *E. lineatus* by Gąsiorek et al. 2019a). In contrast, males of *E. masculinus* sp. nov. and *E. clevelandi* can be easily distinguished based on the differences in dorsal sculpturing and appendage configuration (compare Beasley 1999 and the present study). Consequently, a question arises: why do females of the *virginicus* complex tend to diverge morphologically at a slower rate than males? The acquisition of genetic data for *E. clevelandi* and *E. hoonsooi* could help to resolve this conundrum, as the putative, basal, character of *E. clevelandi* and *E. masculinus* sp. nov. within the *virginicus* clade would support the hypothesis that asexually reproducing species are young and poorly phenotypically differentiated from each other and from the ancestral female phenotype. Finally, considering that the sexually reproducing *E. masculinus* sp. nov. is a sister taxon to the asexual *E. lineatus* + *E. virginicus* clade, we hypothesise that the males were originally present in the ancestor of the clade. Moreover, given the overall similarity of males of *E. clevelandi* and *E. masculinus* sp. nov., we also hypothesise that males in the ancestral lineage leading to *E. lineatus* and *E. virginicus* were phenotypically similar to males of *E. masculinus* sp. nov.

Conclusions

The description of sexually dimorphic *E. masculinus* sp. nov. elucidates the evolution of the *virginicus* complex and raises new questions about the phenotype evolution in tardigrades. Females of three species (*E. lineatus*, *E. virginicus* and *E. masculinus* sp. nov.) represent an exemplary case of delusively similar taxa (i.e. almost identical under PCM but easily identifiable with SEM analysis). The tardigrade fauna of the Indomalayan region requires more sampling effort to uncover its diversity and uniqueness.

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Supplementary material 1

Raw morphometric data for the type population

Authors: Piotr Gąsiorek, Katarzyna Vončina, Łukasz Michalczyk

Data type: morphometric data

Explanation note: The dataset comprises individual measurements provided separately for all life stages.

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Link: <https://doi.org/10.3897/zse.96.49989.suppl1>

Supplementary material 2

Uncorrected pairwise distances

Authors: Piotr Gąsiorek, Katarzyna Vončina, Łukasz Michalczyk

Data type: genetic data

Explanation note: p-distances between haplotypes of fastly evolving DNA fragments (ITS-1, ITS-2, COI) provided for the members of the *virginicus* complex.

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